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Spatial patterns of FUS-immunoreactive neuronal cytoplasmic inclusions (NCI) in neuronal intermediate filament inclusion disease (NIFID)

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Abstract

Neuronal intermediate filament inclusion disease (NIFID), a rare form of frontotemporal lobar degeneration (FTLD), is characterized neuropathologically by focal atrophy of the frontal and temporal lobes, neuronal loss, gliosis, and neuronal cytoplasmic inclusions (NCI) containing epitopes of ubiquitin and neuronal intermediate filament (IF) proteins. Recently, the 'fused in sarcoma' (FUS) protein (encoded by the *FUS* gene) has been shown to be a component of the

inclusions of NIFID. To further characterize FUS proteinopathy in NIFID, we studied the spatial patterns of the FUS-immunoreactive NCI in frontal and temporal cortex of 10 cases. In the cerebral cortex, sectors CA1/2 of the hippocampus, and the dentate gyrus (DG), the FUS-immunoreactive NCI were frequently clustered and the clusters were regularly distributed parallel to the tissue boundary. In a proportion of cortical gyri, cluster size of the NCI approximated to those of the columns of cells was associated with the cortico-cortical projections. There were no significant differences in the frequency of different types of spatial patterns with disease duration or disease stage. Clusters of NCI in the upper and lower cortex were significantly larger using FUS compared with phosphorylated, neurofilament heavy polypeptide (NEFH) or α -internexin (INA) immunohistochemistry (IHC). We concluded: (1) FUS-immunoreactive NCI exhibit similar spatial patterns to analogous inclusions in the tauopathies and synucleinopathies, (2) clusters of FUS-immunoreactive NCI are larger than those revealed by NEFH or IMA, and (3) the spatial patterns of the FUS-immunoreactive NCI suggest the degeneration of the cortico-cortical projections in NIFID.

Keywords

Neurofilament intermediate filament inclusion disease (NIFID); 'Fused in sarcoma' (FUS); Neuronal cytoplasmic inclusions (NCI); Spatial pattern; Cortico-cortical projections

Introduction

Neuronal intermediate filament inclusion disease (NIFID), a rare form of frontotemporal lobar degeneration (FTLD), is characterized by an early-onset and a variable phenotype that includes frontotemporal dementia (FTD), pyramidal, and extrapyramidal signs (Bigio et al. 2003, Cairns et al. 2003, Josephs et al. 2003). Neuropathologically, there is degeneration of the cerebral cortex, striatum, and brain stem with neuronal loss in the frontal, parietal, and temporal cortex (Bigio et al. 2003, Cairns et al. 2003, Cairns et al. 2004a, Armstrong et al. 2006). Abnormal aggregates of neuronal intermediate filament (IF) proteins in the form of neuronal cytoplasmic inclusions (NCI) have been regarded as the pathological 'signature' of NIFID (Cairns et al. 2004b). In addition, abnormally enlarged neurons (EN) and a reactive gliosis are observed in the affected areas (Armstrong et al. 2006, Cairns et al. 2003).

The spatial patterns of the pathological changes in the frontal and temporal lobe have been quantified previously in NIFID using antibodies that recognize either a phosphorylated epitope of neurofilament heavy polypeptide (NEFH) (Cairns and Armstrong 2003, Cairns et al. 2004a) or the intermediate filament (IF) protein α -internexin (INA) (Armstrong and Cairns 2006a, b, 2007, Armstrong et al. 2006), one of the four proteins that comprise the type IV IF proteins (Ching and Liein 1998, Ching et al. 1999). Not all inclusions in NIFID, however, are immunolabelled by anti-NEFH or neuronal IF immunohistochemistry (IHC), and therefore, the primary molecular defect remains uncertain (Neumann et al. 2009a). Recently, the 'fused in sarcoma' (FUS) protein (encoded by the *FUS* gene) has been shown to be a component of the inclusions of familial amyotrophic lateral sclerosis (ALS) with *FUS* mutation (Kwiatkowski et al. 2009, Valdmanis et al. 2009, Vance et al. 2009), and three FTLD entities, viz., NIFID (Neumann et al. 2009a, Armstrong et al. 2010), basophilic inclusion body disease (BIBD) (Munoz et al. 2009), and atypical FTLD with ubiquitin-immunoreactive inclusions (aFTLD-U) (Neumann et al. 2009b).

In the cerebral cortex of disorders with tau-immunoreactive inclusions (tauopathies), e.g., Alzheimer's disease (AD) (Armstrong 1993a), Pick's disease (PiD) (Armstrong et al. 1998), corticobasal degeneration (CBD) (Armstrong et al. 1999), and progressive supranuclear palsy (PSP) (Armstrong et al. 2007), and in the synucleinopathies, dementia with Lewy bodies

(DLB) (Armstrong et al. 1997) and multiple system atrophy (MSA) (Armstrong et al. 2004), the cellular inclusions have a characteristic spatial pattern, viz., they occur in distinct clusters which are regularly distributed parallel to the pia mater (Armstrong et al. 2001). In addition, inclusions in NIFID revealed by NEFH (Cairns and Armstrong 2003) and IMA (Armstrong and Cairns 2006a) IHC exhibit a similar spatial pattern. These results suggested that the inclusions developed in relation to clusters of cells associated with the cortico-cortical and cortico-hippocampal projections and may spread between cortical regions via these connections (Goedert et al. 2010). Hence, the objectives of the present study were to determine whether: (1) the FUS-immunoreactive NCI in NIFID exhibit a similar pattern of topographic distribution, (2) the spatial pattern is related to disease duration or stage, and (3) there are differences in spatial patterns revealed by FUS compared with NEFH and INA IHC.

Materials and methods

Cases

Ten well-characterized cases of NIFID (see Table 1) obtained from Canada, Norway, Spain, Japan (one case from each), and from France, the UK, and the USA (two cases from each) were studied and have been described in detail by Cairns et al. (2004c). Patients had no family history of psychiatric or neurological disorders. Presenting symptoms included personality change, apathy, blunted affect, and disinhibition in seven patients and memory loss and cognitive impairments in five patients. Motor weakness was evident at presentation in three patients and extrapyramidal features in eight patients. All cases displayed the previously defined histological features of NIFID, viz., IMA-immunoreactive NCI, together with EN and gliosis in the cerebral cortex and striatum (Cairns et al. 2004c). None of the cases had pathological features immunoreactive for phosphorylated tau, a cytoskeletal protein specific for astrocytes, glial fibrillary acidic protein (GFAP) (Cairns et al. 2004a, c), or microglia (CD68) (Cairns et al. 2004a,c), prion protein, α -synuclein (Cairns et al. 2004b), or TDP-43 (Armstrong et al. 2010) thus eliminating a diagnosis of a tauopathy, synucleinopathy, prion disease, or TDP-43 proteinopathy. The degree of brain atrophy varied between cases and was related to the four-stage scheme for severity of atrophy in FTLN (Broe et al. 2003, Armstrong et al. 2006). In this scheme, case G had the least (stage 1) and cases A, B and I had the most severe atrophy (stage 3). The remaining cases had intermediate levels of atrophy (stages 2).

Histological methods

After death, the consent of the next of kin was obtained for brain removal, following local Ethical Committee procedures and the 1995 Declaration of Helsinki (as modified Edinburgh, 2000). Brain tissue was preserved in buffered 10% formalin or 4% paraformaldehyde. Tissue blocks were taken from the frontal cortex at the level of the genu of the corpus callosum to study the superior frontal cortex (SFC), and temporal lobe at the level of the lateral geniculate body. Within the temporal lobe, the superior temporal gyrus (STG), parahippocampal gyrus (PHG), CA sectors of the hippocampus (HC) (CA1/CA2), and the dentate gyrus (DG) were studied. Tissue was fixed in 10% phosphate buffered formal-saline and embedded in paraffin wax. Following microwave pretreatment, IHC was performed on 6–8 μ m sections with a commercial anti-FUS rabbit poly-clonal antibody (1:1,500; Sigma-Aldrich, St. Louis, MO, USA). Sections were counterstained with haematoxylin and were taken from the same tissue blocks adjacent to those previously studied using NEFH and INA IHC (Armstrong et al. 2006).

Morphometric methods

In each cortical gyrus, histological features were quantified along a strip of tissue (3,200–6,400 μm in length) located parallel to the pia mater, using $250 \times 50 \mu\text{m}$ sample fields arranged contiguously. The sample fields were located both in the upper cortex (approximating to laminae II/III) and lower cortex (approximating to laminae V/VI), the short edge of the sample field being orientated parallel with the pia mater and aligned with guidelines marked on the slide. In the HC, the histological features were counted from sector CA1 to CA2, the short dimension of the contiguous sample field being aligned with the alveus. Pathological changes were also observed in the DG granule cells (Cairns et al. 2004c, Armstrong et al. 2006) and to quantify these lesions, the sample field was aligned with the upper edge of the granule cell layer.

Data analysis

To determine the spatial patterns of the NCI, the data were analyzed by spatial pattern analysis (Armstrong 1993b, 1997, 2006). This method uses the variance-mean ratio (V/M) to determine whether the NCI were distributed randomly ($V/M = 1$), regularly ($V/M < 1$), or were clustered ($V/M > 1$) along a strip of tissue. Counts of NCI in adjacent sample fields were added together successively to provide data for increasing field sizes, e.g., $50 \times 250 \mu\text{m}$, $100 \times 250 \mu\text{m}$, $200 \times 250 \mu\text{m}$ etc., up to a size limited by the length of the strip sampled. V/M was then plotted against the field size to determine whether the clusters of NCI were regularly or randomly distributed and to estimate the mean cluster size parallel to the tissue boundary. A V/M peak indicates the presence of regularly spaced clusters while an increase in V/M to an asymptotic level suggests the presence of randomly distributed clusters. The statistical significance of a peak was tested using the ' t ' distribution (Armstrong 1997). To examine the relationship between the spatial pattern, disease duration, and disease stage (Broe et al. 2003), variables were divided into categories and the proportions of random, regular, and clustered distributions exhibited by the FUS-immunoreactive inclusions compared using chi-square (χ^2) contingency tables. Cluster sizes of the NCI in the upper and lower cortex revealed by FUS were compared with those revealed by NEFH based on four cases of NIFID (Cairns and Armstrong 2003) and INA IHC based on all ten cases (Armstrong and Cairns 2006a) using two-factor, split-plot analysis of variance (ANOVA). In addition, the degree of correlation between the cluster sizes revealed by FUS and INA IHC and disease duration, brain weight, and disease stage were tested using Pearson's correlation coefficient (' r '). There were insufficient data to test correlations using the NEFH preparations.

Results

Examples of the FUS-immunoreactive NCI in the superficial laminae of the SFG shown in Fig 1. The NCI were commonly round, oval, or cup-shaped, but the other morphologies were present including crescent and tangle-shaped inclusions.

Examples of the spatial patterns shown by the FUS-immunoreactive NCI are shown in Fig 2. In the lower laminae of the PHG (Case A), there was a V/M peak at a field size of $100 \mu\text{m}$ suggesting a regular distribution of clusters of NCI parallel to the pia mater. In the upper laminae of the ITG (Case A), the V/M ratio was significantly below unity at all field sizes suggesting a uniform or regular distribution of the NCI. In the DG (Case A) there was an increase in V/M with field size suggesting the presence of large clusters of NCI of at least $800 \mu\text{m}$ in diameter.

The spatial patterns exhibited by the NCI in each brain region of each case are summarized in Table 2. In the cerebral cortex, the NCI were distributed in clusters ($50\text{--}400 \mu\text{m}$) which

were regularly distributed parallel to the pia mater in 19/44 (43%) of gyri studied, a random distribution of NCI was present in 6/44 (14%) gyri, a uniform distribution in 11/44 (25%) gyri, and NCI were present in larger, non-regularly distributed clusters ($\geq 400 \mu\text{m}$) in 8/44 (18%) gyri. In sectors CA1/2 of the hippocampus and in the DG, regularly distributed clusters of inclusions were present in 6/14 (43%) regions studied. In the data as a whole, clustering of NCI at two scales i.e., where smaller-sized clusters of NCI were aggregated into larger clusters, was present in only a single cortical region. In laminae II/III of cortical gyri exhibiting clustering of NCI, clusters were in the size range $400\text{--}800 \mu\text{m}$ in 5/12 (42%) gyri, clusters were smaller than $400 \mu\text{m}$ in 6/12 (50%) gyri, and clusters were greater than $800 \mu\text{m}$ in 1/12 (8%) gyri. In laminae V/VI, cluster sizes of NCI were $400\text{--}800 \mu\text{m}$ in 6/15 (40%) gyri, smaller than $400 \mu\text{m}$ in 6/15 (40%) gyri, and greater than $800 \mu\text{m}$ in 3/15 (20%) gyri. The distribution of spatial patterns was similar in the cortical regions, CA1/2, and in the DG ($\chi^2 = 1.28$, 3DF, $P > 0.05$). There were no significant differences in the proportions of the different spatial patterns in cases of different disease duration ($\chi^2 = 7.11$, 6DF, $P = 0.05$) or with the degree of brain atrophy ($\chi^2 = 14.83$, 9DF, $P > 0.05$). Correlations between the cluster size of NCI and disease duration, brain weight and disease stage are shown in Table 3. There were no significant correlations between the cluster size of FUS-immunoreactive NCI and these variables, but the cluster size of the INA-immunoreactive NCI were positively correlated with disease duration in the DG and with disease stage in the upper laminae of the PHG.

A comparison of the cluster sizes of NCI revealed by FUS, NEFH, and INA IHC is shown in Fig 3. There were highly significant differences in cluster size between the three antibodies ($F = 20.80$, $P < 0.001$) with significantly larger cluster sizes revealed by FUS compared with NEFH and IMA IHC. In addition, cluster sizes of the NCI were similar in the upper and lower cortex ($F = 0.50$, $P > 0.05$) and the differences in cluster size revealed by the three antibodies were also similar in the upper and lower cortex ($F = 3.08$, $P > 0.05$).

Discussion

NIFID is a rare disorder with considerable variability in pathology between individuals and brain regions. However, in the majority of brain regions studied, the FUS-immunoreactive NCI were present in clusters that were regularly distributed parallel to the tissue boundary. This spatial pattern is similar to that reported for various cellular inclusions in the tauopathies (Armstrong 1993b; Armstrong et al. 1998, 1999, 2007), synucleinopathies (Armstrong et al. 1997, 2004) and in NIFID revealed by NEFH (Cairns and Armstrong 2003) and IMA IHC (Armstrong and Cairns 2006a, b) suggesting a common pattern of cortical degeneration in these disorders.

The spatial pattern of the inclusions within the cerebral cortex and hippocampus suggests a possible relationship with the cells of origin of specific cortico-cortical and cortico-hippocampal projections (De Lacoste and White, 1993; Hiorns et al. 1991; Delatour et al. 2004). The cells of origin of these projections are clustered and occur in bands that are regularly distributed along the cortex parallel to the pia mater. In addition, individual bands of cells, approximately $500\text{--}800 \mu\text{m}$ in width, traverse the laminae in columns (Hiorns et al. 1991). In 19 cortical gyri studied in NIFID, the clusters of NCI were regularly distributed parallel to the pia mater consistent with their development in relation to these connections. In 11 of these gyri, the estimated width of the clusters of FUS-immunoreactive NCI was $400\text{--}800 \mu\text{m}$ approximating to the dimension of the clusters of cells of origin of the cortico-cortical projections (Hiorns et al. 1991). In many remaining cortical gyri, however, the NCI were present in smaller clusters, $50\text{--}200 \mu\text{m}$ in diameter. Furthermore, in one cortical gyrus, the smaller clusters of NCI were aggregated into larger 'superclusters' and in a further four gyri, clusters were larger than $800 \mu\text{m}$. Hence, the pathology may initially involve a subset

of neurons within a column but as the disease develops, it may spread to affect more of the column and cells in adjacent columns (Armstrong et al. 2001). Consistent with this suggestion, recent research suggests that there may be a mechanistic link between different cell populations with intercellular transport of pathological proteins (Goedert et al. 2010). Nevertheless, the type of spatial pattern exhibited was not statistically related to disease duration or disease staging suggesting that there was no simple relationship between the clustering of FUS-immunoreactive NCI and the developing disease. However, the sample size may have been too small to demonstrate the significant correlations with these variables and in addition, there was only one case with a disease duration >5 years while eight other cases had durations between 2.7 and 4 years. Nevertheless, a similar lack of correlation has also been observed in the tauopathies AD (Armstrong 1993b), PiD (Armstrong et al. 1998), CBD (Armstrong et al. 1999) and PSP (Armstrong et al. 2007) and in the synucleinopathies DLB (Armstrong et al. 1997) and MSA (Armstrong et al. 2004). By contrast, there were significant positive correlations between the cluster size of the INA-immunoreactive NCI and disease duration or disease stage in some brain areas suggesting that INA-IHC increases during the course of the disease and therefore, that FUS activity may precede that of INA.

Cluster sizes of the NCI in the upper and lower cortex were significantly greater revealed by FUS than by either NEFH or INA IHC. These results are consistent with previous studies (Neumann et al. 2009a, Armstrong et al. (2010) which reveal significantly greater densities of inclusions using FUS compared with IMA. In addition, Neumann et al. (2009a) using double-labeling studies reported that many cells in NIFID had only FUS-immunoreactive inclusions while all cells with IF-immunoreactive inclusions were also labeled by FUS. Hence, despite the fact that, to date, no mutations in the *FUS* gene have been found in NIFID (Neumann et al. 2009a), FUS could play a significant role in the pathogenesis of NIFID (Neumann et al. 2009a).

In conclusion, NIFID is a rare disorder and one of the several diseases that constitute the FTL-D-FUS proteinopathies. Neuropathologically, NIFID is characterized by widespread degeneration of the frontal and temporal lobes with significant densities of FUS-immunoreactive NCI in these regions. In many regions, the FUS-immunoreactive NCI are distributed in clusters which are regularly distributed parallel to the tissue boundary. In this relatively small group of cases, the cluster size of the FUS-immunoreactive NCI is significantly greater than revealed by either NEFH or IMA. These results suggest a significant role for FUS in the pathogenesis of NIFID.

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Fig. 1. Neuronal cytoplasmic inclusions (NCI) (*arrows*) in the superficial laminae of the frontal lobe ($\times 200$) in neuronal intermediate filament inclusion disease (NIFID). (FUS immunohistochemistry, *bar* 25 μm)

Fig. 2.
Examples of the spatial patterns exhibited by FUS-immunoreactive neuronal cytoplasmic inclusions (NCI) in a case of neuronal intermediate filament inclusion disease (NIFID) (Case A), ** significant variance/mean peak

Fig. 3.

A comparison of the cluster sizes of the FUS-immunoreactive neuronal cytoplasmic inclusions (NCI) with those revealed using neurofilament, heavy polypeptide (NEFH) and α -internexin (INA) immunohistochemistry (IHC) in the *upper* (II/III) and *lower* (V/VI) laminae in areas of cerebral cortex in neuronal intermediate filament inclusion disease (NIFID). Analysis of variance (ANOVA): Between antibodies $F = 20.80$ ($P < 0.001$); Between *upper* and *lower* cortex $F = 0.50$ ($P > 0.05$); Interaction $F = 3.08$ ($P > 0.05$)

Table 1
Demographic features, gross brain weight, and disease stage of the neuronal intermediate filament inclusion disease (NIFID) cases studied

Case	Sex	Age at onset (years)	Age at death (years)	Duration (years)	Brain weight (g)	Stage
A	F	23	28	5	860	3
B	F	25	29	4	710	3
C	M	32	35	3	NA	2
D	F	38	41	3	904	2
E	M	39	42.5	3.5	950	2
F	M	47	50	3	1,200	2
G	M	48	52	4	1,310	1
H	M	48	61	13	850	2
I	F	52	54.7	2.7	813	3
J	M	56	60	4	1,250	2

M Male, F female, NA data not available

Table 2
Spatial pattern of the FUS-immunoreactive neuronal cytoplasmic inclusions (NCI) in areas of the frontal and temporal lobe in 10 cases of neuronal intermediate filament inclusion disease (NIFID)

Brain region and lamina											
Case	SFG	II/III	V/VI	ITG	II/III	V/VI	PHG	II/III	V/VI	HC	DG
A	RG		>400	RG		>400	400		100	100	>800
B	400		RG	RG		100	RG		400	RG	RG
C	200		400	-		-	-		-	-	-
D	R		R	400		>800	RG		50	R	RG
E	RG		R	-		-	-		-	-	-
F	>400		-	RG		RG	>800		>400	400	400
G	400		RG	-		200	100		50	200	100
H	50,200		R	-		-	50		50	400	400
I	200		>800	-		-	-		-	-	-
J	100		>800	R		400	RG		R	R	RG

Data represent the dimensions (μm) measured parallel to the pia mater, alveus or dentate gyrus granule cell layer of regularly distributed clusters of NCI with the following exceptions: *R* Random distribution, *RG* regular distribution of individual inclusions. >800 indicates large scale clustering of inclusions of at least the size listed. Where two figures are present, clustering occurs at two scales, i.e., smaller clusters are aggregated into larger 'superclusters'

SFG superior frontal cortex, *ITG* inferior temporal gyrus, *PHG* parahippocampal gyrus, *HC* hippocampus, *DG* = dentate gyrus
(-) indicates either tissue section unavailable or density of inclusions was too low to determine their spatial pattern

Table 3
Correlations (Pearson's 'r') between the cluster size of the neuronal cytoplasmic inclusions (NCD) revealed by FUS and α -internexin (INA) immunohistochemistry (IHC) and disease duration, brain weight, and disease stage in each brain region in 10 cases of neuronal intermediate filament inclusion disease (NIFID)

Brain region and lamina									
Variable	IHC	SFG II/III	V/VI	ITG II/III	V/VI	PHG II/III	V/VI	HC	DG
Disease	FUS	-0.46	0.43	-	0	-0.63	-0.38	0.40	-0.76
Duration	INA	0.08	-0.34	-0.37	0.72	-0.39	-0.53	0.28	0.96**
Brain weight	FUS	-0.10	0.58	-	0.22	0.36	0.18	-0.27	-0.76
	INA	-0.75	-0.61	0.01	0.71	-0.64	0.18	-0.57	-0.17
Disease stage	FUS	0.32	0.18	-	-0.21	0.22	0.21	0.07	0.04
	INA	0.35	0.23	0.01	-0.38	0.95**	-0.32	-0.12	-0.34

SFG superior frontal cortex, ITG inferior temporal gyrus, PHG parahippocampal gyrus, HC hippocampus, DG dentate gyrus

(-) indicates insufficient data to test correlation

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$P < 0.01$